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Review

The applications of nanopores in studies of proteins

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ABSTRACT

Nanopores are a label-free platform with the ability to detect subtle changes in the activities of individual biomolecules under physiological conditions. Here, we comprehensively review the technological development of nanopores, focusing on their applications in studying the physicochemical properties and dynamic conformations of peptides, individual proteins, protein-protein complexes and protein-DNA complexes. This is followed by a brief discussion of the potential challenges that need to be overcome before the technology can be widely accepted by the scientific community. We believe that with continued refinement of the technology, significant understanding can be gained to help clarify the role of protein activities in the regulation of cellular physiology and pathogenesis.

1. Introduction

Proteins are complex organic molecules that play various roles in maintaining metabolism and regulating physiological functions [1]. Over the past decades, several methods have been developed for protein research [2], including two-dimensional electrophoresis (2-DE) [3], high-performance liquid chromatography (HPLC) [4], mass spectrometry (MS) [5], X-ray crystallography [6], nuclear magnetic resonance (NMR) spectroscopy [7], circular dichroism [8], atomic force microscopy (AFM) [9], optical tweezers [10], fluorescence resonance energy transfer (FRET) [11] and cryoelectron microscopy [12]. Although the aforementioned techniques are widely used and valued in facilitating our understanding of protein structure and function, each technique has its limitations. First, certain methods (2-DE, HPLC, MS, X-ray crystallography, NMR and circular dichroism) require a large amount of identical proteins. Second, it is difficult to capture conformational changes of a single protein in real time. The need to better characterize protein properties and activities has led to the development of nanopores, which range from 1 to 100 nm in size and have the capacity to trace the ionic current passing through them upon application of a voltage [2]. Compared with other technologies, the advantages of nanopore-based methods for protein analysis are (1) their sensitivity to detect subtle changes in single proteins even at very low concentrations; (2) their ability to provide a dynamic view of protein conformation under physiological conditions; and (3) their label-free nature [2,13,14]. These advantages make nanopore technology a practical alternative and perhaps a more suitable approach for protein characterization.

The use of nanopores for biological detection was pioneered by Kasianowicz et al. [15] in 1996. In a typical nanopore experiment, two reservoirs filled with buffer solution are connected only through a nanopore. Positive and negative voltage is applied through electrodes immersed in the buffer solution. As charged analytes translocate through the nanopore under the effect of the electric field, a pulse-resistive-like signal emerges because of the variation in electrical conductivity. In the context of nucleic acid detection, these ionic current signals allow four different nucleotides to be distinguished based on differences in dwell time and blockade current [16]. While nanopores were initially used for DNA sequencing [17,18], increasing evidence has shown that they can be used to analyze the physical and chemical properties of other molecules [19], including but not limited to RNA [20], proteins [21], nanoparticles [22] and metal ions [23,24].

Nanopores can have different measurement capacities based on the materials used and can be divided into two major types: biological nanopores and solid-state nanopores. Biological nanopores are made of proteins with funnel-shaped structures, such as α-hemolysin [25–32], Msp A [18] and Phi29 [33]. Solid-state nanopores are made of nonbiological materials [34,35], such as silicon nitride [36,37], quartz [38], and graphene [39–43]. Currently, both types of nanopores have shown promise in providing critical information regarding protein characteristics using a small amount of protein.

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To date, several review articles discussing the application of nanopores for protein sensing have been published [2,13,14]. For example, key nanopore materials, nanoscale features, and design requirements for detecting and characterizing proteins at the single-molecule level using nanopores have been described [2]. Additionally, recent progress in nanopore-based studies of the translocation features of specific proteins bound to DNA [13] and the various means of identifying peptides, proteins, and their complexes [14] have been discussed. In the present review, we summarize and highlight several areas in the field of nanopore-based protein characterization, including studies of nanopore-based peptide detection and amino acid sequencing, measurement of single protein properties, dynamics of protein folding/unfolding and modifications, and interactions of proteins with other biomolecules (particularly DNA assembled nanostructures).

2. Amino acids and peptides

The amino acid (aa) is the basic unit of protein molecules and the elemental component of metabolism in living organisms. In our bodies, amino acids can be synthesized into functional proteins, such as enzymes and antibodies. While the sequence of amino acids affects the structure and function of proteins. Abnormal amino acids will disturb the normal metabolism in the body and eventually lead to disease [44]. Thus, detection and sequencing of amino acids are very important in biomedical research. Nanopore technology serves as a suitable alternative for the study of amino acids and peptides. Considering that the small size (approximately 1 nm) of the restricted region of biological nanopores approaches the size of amino acids, biological nanopores rather than solid-state nanopores are usually utilized to detect amino acids and peptides.

2.1. Direct identification of amino acids and peptides

Biological nanopores are the most commonly used nanopores for direct identification of amino acids and peptides. As early as 2004, Sutherland et al. [45] identified peptides containing a different number of repeats (Gly-Pro-Pro) using α-HL. Then, in 2006, Stefureac et al. [46] analyzed different α-helical peptides by measuring the current blockade and the duration time. From histograms of the blockade current, $D_2A_{10}K_2$, $D_2A_{14}K_2$, $D_2A_{18}K_2$, $D_2A_{22}K_2$, $D_3A_{14}K_2$ and $DA_{14}K$ were discriminated using α-HL and aerolysin pores. One year later, Goodrich et al. [47] explored the difference in the translocation signals of various β-hairpin peptides using α-HL. Through electrical recordings and molecular dynamics simulations, they found that unfolded peptides translocated the nanopore faster than folded peptides. In the early stage, researchers typically focused on discrimination of peptides with a various number or type of amino acids. In 2010, Bikwemu et al. [48] studied the kinetics of polypeptide translocation. They set a kinetic model to realize quantitative assessment of the exit frequency of a polypeptide through a protein nanopore. In the same year, Meng et al. [49] analyzed tethered peptides. They linked 12-aa-long peptides to mono-, di-, tri-, and tetrabromomethyl-substituted benzene to form peptide bundles (CY_{12}-T_1 to CY_{12}-T_4). From histograms of the blockade current and duration time, they found that CY_{12}-T_1 and CY_{12}-T_2 only gave rise to bumping and translocation events, while CY_{12}-T_3 and CY_{12}-T_4 gave rise to intercalation events. In 2011, Wang et al. [50] investigated the aggregation states of amyloid-β 42 (Aβ42) in the presence of a promoter (β-cyclodextrin) or inhibitor (Congo red) through statistical analyses of blockade current and duration time. In 2012, Boersma and Bayley [51] successfully discriminated amino acid enantiomers with an α-HL nanopore. In their experiments, the protein nanopore was covalently modified with iodoacetamide containing phenanthroline at Cys-117. Copper ions were added and linked to the phenanthroline. Then, D-amino acids or L-amino acids could interact with the copper ion, leading to different conductance variations. Finally, they discriminated several pairs of amino acid enantiomers using this method. In 2014, Mereuta et al. [52] reported how peptides translocate through an α-HL nanopore based on a pH-tuned method and molecular dynamics (MD) simulations. They observed intermediate substrates of translocation signals under various pH values. Similarly, in 2016, Asandei et al. [53] studied the passage of a single peptide through α-HL under various voltages and pH conditions. In 2017, Asandei et al. [54] reported the ability of α-HL to distinguish different neutral amino acids based on the fluctuation in ionic current. Specifically, the feature information of engineered polypeptide chains containing 30 alanine or 30 tryptophan residues was extracted from the ionic current fluctuations to achieve discrimination. In the same year, Chavis et al. [55] utilized single-molecule nanopore spectrometry (SMNS) to discriminate a variety of peptides through correlations between current blockade values and the mass of the peptides. In 2019, Larimi et al. [56] found that the translocation of a single polypeptide was substantially dependent on the concentrations of polyethylene glycols (PEGs).

We should also mention that peptide translocation through a nanopore can be assisted by the use of facilitator molecules, such as DNA. For example, in 2012, Ying et al. [57] studied the structural properties of two peptide-oligonucleotide conjugates using α-HL. The current signature of the conjugates could be divided into three stages: a capture stage, unfolding stage and translocation stage. The blockade current and dwell time of different conjugates were distinguishable. In 2018, Ciuca et al. [58] designed and prepared peptide nucleic acids (PNAs) to facilitate detection of peptides and DNA. In their experiment, the opposite charged peptides and DNA would separate to thread through the nanopore under the influence of an electric field.

2.2. Proteolysis-based peptide detection

Similar to applications of cleavage-based protocols in DNA sequencing, proteolysis-based methods are also utilized to improve the resolution of nanopores to the single amino acid level in peptide detection. In 2009, Zhao et al. [59] monitored the cleavage of a peptide in real time using engineered α-HL. In their experiments, Aβ peptide was treated with a protease (trypsin). With prolongation of the reaction time, the percentage of digested peptide increased, which could be calculated from the current traces and amplitude histograms. In 2015, Wang et al. [60] reported nanopore-based detection of botulinum neurotoxins (BoNTs). BoNTs can digest synaptobrevin into two peptides, which can be sensed. Through the parameters of ionic current signals, rapid and highly sensitive detection of neurotoxins was realized. Later, in 2018, Piguet et al. [61] distinguished short homopeptides in a mixture at single amino acid resolution (Fig. 1). In their experiments, the length and type of the polypeptide could be inferred from statistical analysis of the dwell time and blockade current. They first detected a mixture of arginine peptides of different lengths and found that both the dwell time and blockage current increased as the length of the arginine peptides increased from 5 to 10. They also treated arginine peptide and lysine peptide with trypsin, separately. Through real-time monitoring of enzymatic degradation of the sample, they observed that the current peaks for long peptides (9-aa-long arginine peptide and 10-aa-long lysine peptide) disappeared gradually as time progressed. In addition, nanopores can be utilized to discriminate 10-aa-long peptides with different sequences. For instance, RR-10 (RRRRRRRRRR),

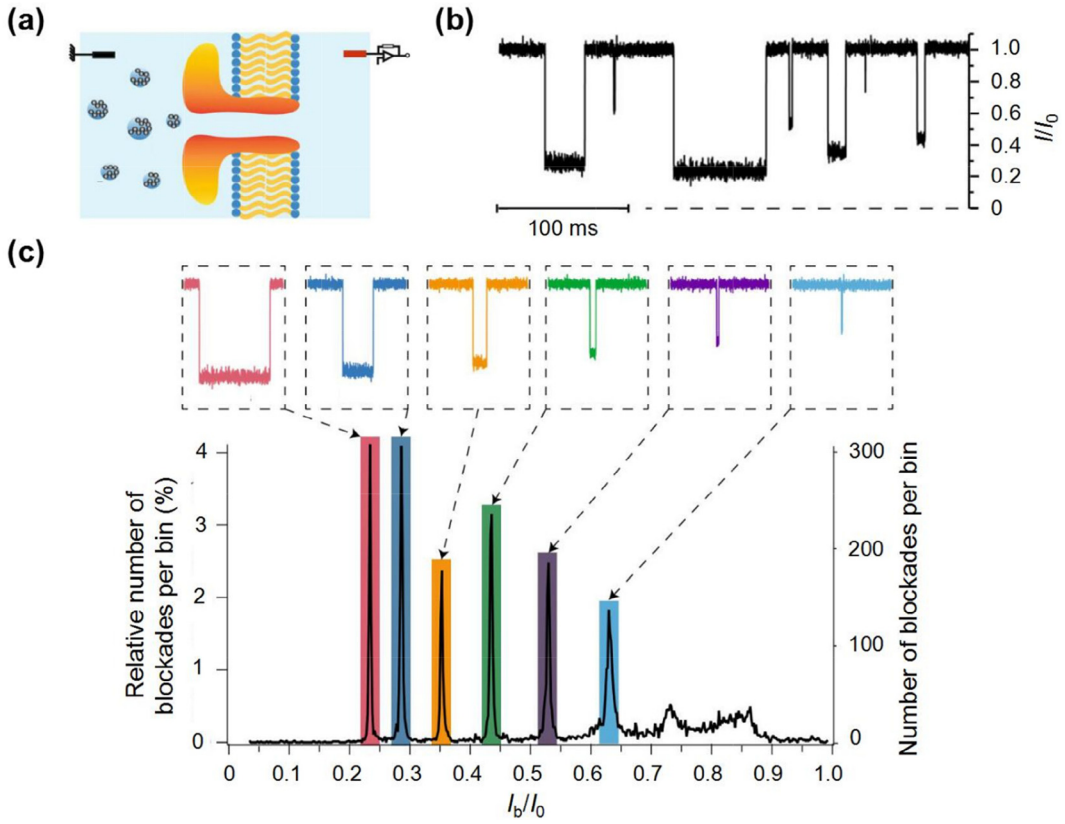

Fig. 1. Discrimination of peptides in a mixture. (a) Detection schematic diagram. (b) Example of an ionic current trace. (c) Current blockade peaks corresponding to peptides of different lengths. Reproduced with permission from Ref. [61]. Copyright 2018, Nature Publishing Group.

KR-10 (KKKKKRRRRR) and KK-10 (KKKKKKKKKK) had significantly different current signatures.

2.3. Characterization of peptide-metal ion binding

Conformational changes in peptides induced by the binding of metal ions have also been characterized using nanopores. In 2010, Stefureac et al. [62] analyzed interactions between metal ions and prion proteins/peptides. They demonstrated that the addition of Cu^{2+}, Zn^{2+}, Mg^{2+} and Mn^{2+} can modulate the conformation of the prion protein (PrP) and prion peptides, which can be observed from histograms of bumping events with α-HL. In 2012, Mereuta et al. [63] investigated the Cu^{2+}-triggered conformational change in a chimeric peptide using α-HL. The 20-aa-long chimeric peptide contained a His residue, which can bind to metal ions. The ionic current signals showed that the association rate constant between the α-HL pore and a Cu^{2+}-free peptide was higher than that of a Cu^{2+}-containing peptide. In 2014, Asandei et al. [64] explored the effect of metal ions on the Aβ peptide based on α-HL. It was found that Cu^{2+}, Zn^{2+}, Fe^{3+}, Al^{3+} can all cause conformational changes in the Aβ peptide. In addition, the propensity of different metal ions to interact with the peptide was in the order Cu^{2+}> Zn^{2+}> Fe^{3+}> Al^{3+}. In 2017, Roozbahani et al. [65] developed a sensor for uranyl (UO_2^{2+}) ions by analyzing the translocation signals of a peptide probe that could specifically bind to uranyl ions.

3. Proteins

Polypeptides are folded and modified to form proteins with specific spatial structures and functions. Using nanopores, we can directly characterize single proteins, study their folding and unfolding processes and detect various protein modifications. All these topics are fundamentally important in protein-related research.

3.1. Direct characterization of native proteins using nanopores

Obtaining the physical and chemical properties of a protein is crucial in understanding its physiological functions. Below, several studies that have employed single nanopores or nanopore arrays for deciphering the size, charge and conformation of specific proteins are described.

In 2007, Fologea et al. [66] studied the charge variations of bovine serum albumin (BSA) under different pH conditions. By analyzing the amplitude, duration and integral of current signals, the relative charge and size of single proteins under the different conditions could be determined. According to the nanopore translocation signatures, it was found that BSA was negatively charged at pH 7 but positively charged at pH 4.5. In 2008, Stefureac et al. [67] used biological nanopores to compare the ionic current signals of wild-type *E. coli* histidine-containing protein (HPr) and mutant HPr. Because of the differences in charge between the wild-type proteins and mutant proteins, molecules with different mutations could be discriminated successfully according to the electrical signals. In 2010, Firnkes et al. [68] studied the translocation of avidin by measuring the nanopore zeta potential and the zeta potential of the protein under different pH. The results showed that the translocation behavior of avidin, including its direction, frequency and duration time, can be affected by the difference between the nanopore zeta potential and the protein zeta potential. In 2012, the same group detected single proteins using chemically modified silicon nitride nanopores. The gold-coated SiN nanopore was functionalized with multivalent nitrilotriacetic acid (NTA), which can interact with His-tagged proteins in the

presence of Ni^{2+}. By this method, subclasses of IgG antibodies could be discriminated based on their current signatures [69]. In 2014, Qiu and Skafida [70] theoretically simulated the conformational change in a single protein passing through a multilayer graphene nanopore. This work evaluated how Angiotensin II proteins with different conformations affect the conductance of the multilayer graphene nanopore, entirely based on molecular dynamics (MD) simulations. In 2017, Waduge et al. [71] employed solid-state nanopores to discriminate the size and structural conformation of several types of proteins according to their ionic current signals. Additionally, the conformational change in calmodulin with and without calcium chloride was also detected (Fig. 2). In 2017, Yusko et al. [72] demonstrated the ability of bilayer-coated solid-state nanopores to characterize single proteins, providing estimations of parameters such as volume, shape, charge, dipole moment and rotational diffusion coefficient, for individual proteins based on current peaks. Furthermore, in 2019, the same group [73] utilized a solid-state nanopore without modification of the bilayer to characterize several parameters (including volume, shape and dipole moment) of various proteins.

Apart from investigation of the physical and chemical properties, understanding a protein's translocation process is also a subject of active research. Currently, several strategies that control the movement of proteins through a nanopore have been described. For example, Mohammad et al. [74] controlled the movement of proteins by introducing electrostatic traps into α-HL. Then, Wu et al. [75] speculated on the translocation process of BSA by examining the local details of translocation signals in 2014. Additionally, in 2015, Biesemans et al. [76] linked two protein molecules using rotaxane and demonstrated the ability to move the proteins back and forth through a nanopore with alternating voltage. In addition, it was shown that the complex could dissociate when the electric field force was sufficiently strong. Furthermore, several studies concerning protein manipulation based on theoretical calculations or MD simulations have also been published [77–79]. These results provide guidance for controlling protein translocation and improving the performance of nanopore-based protein detection.

In addition to single nanopores, nanopore arrays can also be applied to characterize the physicochemical properties of proteins through electrical or optical methods. For instance, Im et al. [80] assembled α-HL on a nanopore array and carried out kinetic binding assays based on surface plasmon resonance (SPR). Through the changes in the transmission SPR spectra, they were able to monitor the incorporation of α-HL. However, compared with single nanopore-based methods, nanopore array-based methods usually lack single-molecule resolution and sensitivity. Moreover, to produce a clear effect on the sensors, this method requires a large number of identical proteins, which might not be feasible in many situations.

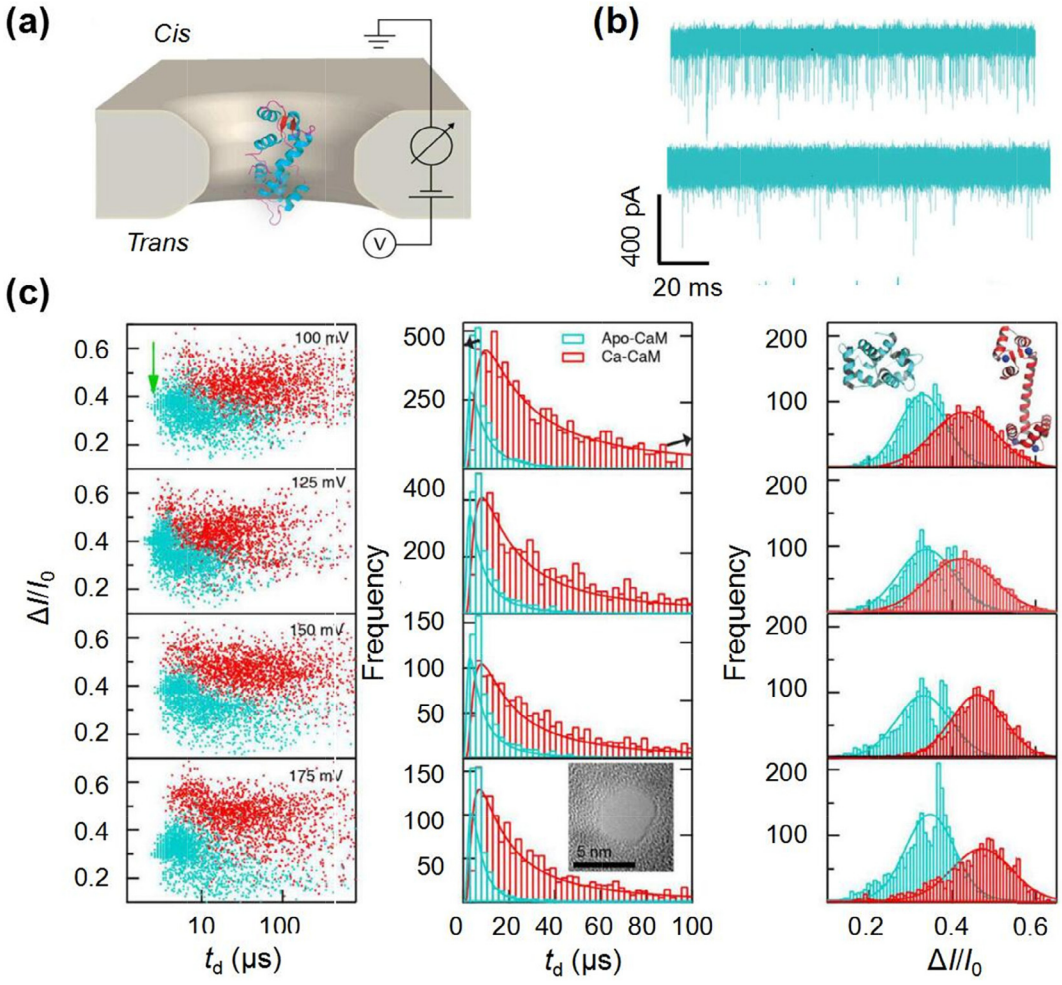

Fig. 2. Direct characterization of single proteins using nanopores. (a) Schematic diagram of protein detection using a solid-state nanopore. (b) Ionic current traces using nanopores with different diameters. (c) Statistical analysis of Apo-CaM and Ca-CaM data, allowing them to be distinguished. Reproduced with permission from Ref. [71]. Copyright 2017, American Chemical Society.

3.2. Single-protein folding and unfolding processes

Folding of linear polypeptides into 3D structures is a crucial step of the protein maturation process, and errors can lead to irreversible biological consequences, such as diseases. To date, many researchers have investigated diseases that are caused by protein misfolding. The topics include: (1) Alzheimer's disease (AD)-related amyloid fibrils formed by misfolding and aggregation of Aβ peptides [81,82]; (2) Parkinson's disease (PD)-related Lewy bodies consisting of α-synuclein [83,84]; and (3) a wide variety of transmissible spongiform encephalopathies (TSEs) caused by misfolding of prion protein [85,86]. However, such studies remain difficult to carry out due to the complexities of the dynamic protein folding and unfolding processes. As an ultrasensitive and label-free alternative method, nanopore technology can provide a dynamic survey of the folding/unfolding process of single proteins with various structures at the same time. In addition, nanopore-based characterization can be performed at relatively low concentrations, thus allowing the dynamics of aggregation to be easily captured since aggregates can form slowly [87]. By extracting the features of dwell time and blockade current from the resulting electrical signatures, a better understanding of the folding and unfolding mechanisms of a single protein can in principle be obtained.

To date, several studies have investigated the protein unfolding process by using maltose binding protein (MBP) and guanidium-HCl (Gdm-HCl). For example, in 2012, Cressiot et al. [88] reported experimental and theoretical findings regarding the transport of an unfolded protein through solid-state nanopores. They unfolded MBP using Gdm-HCl and compared the difference in electrical parameters under 200 and 750 mV. Similarly, in the same year, Merstorf et al. [89] used a nanopore as a platform to explore the folding and unfolding transition (Fig. 3a, b). In their experiments, MBP was again treated with Gdm-HCl. From the ionic current traces, they could distinguish unfolded MBP from partially folded MBP by using an aerolysin pore or α-HL. Differences between the unfolding transition curves for native and variant proteins were also analyzed. Moreover, in 2014, Pastoriza-Gallego et al. [90] observed the translocation of a chimeric molecule through an aerolysin nanopore in the presence of Gdm-HCl. The chimeric molecule was composed of covalently linked recombinant MBP and ssDNA. They compared the ionic current signals of the chimera, MBP and the oligonucleotide to confirm the translocation of unfolded proteins.

Urea has also been used to unfold proteins. In 2013, Freedman et al. [91] compared the dwell time and blockade current of unfolded SAP97 PDZ2 under different voltages. By comparing two variants of the protein, voltage-dependent unfolding was found to correlate with the protein's stability. In the same year, Rodriguez-Larrea and Bayley [92] revealed the mechanism of unfolding during protein translocation through a narrow biological nanopore (Fig. 4a). Using thioredoxin, they found evidence supporting a four-step translocation model based on the ionic current trace (Fig. 4b, c). The ratio of one-step events (indicating translocation of unfolded proteins) increased as the concentration of urea increased.

Other methods, such as the use of unfoldases, heating or MD simulations, have also been employed to study the protein unfolding process. In 2013, Akeson's group [93] realized the unfolding and translocation of individual proteins through α-HL with the assistance of the unfoldase ClpX. Based on this work, the group showed in 2014 that the unfoldase ClpX protein could facilitate discrimination of protein variants using α-HL [94]. In their scheme, the domains of proteins unfolded in a specific order and translocated through nanopores assisted by unfoldase and voltage, providing a means to identify protein domains and structural modifications at the single-molecule level from the local details of electrical signatures. Using the heating approach, Dong et al. [95] unfolded a single protein by applying heat and stabilized it with sodium dodecyl sulfate (SDS) and β-mercaptoethanol (BME) in 2017. This AFM and nanopore-based method was sufficiently sensitive to distinguish amino acid substitutions in a single unfolded protein. Using a simulation approach, Xu et al. [96] investigated the unfolding process of ubiquitin in 2016. They compared the unfolding process using nanopores with the unfolding process using AFM. In 2017, Si and Aksimentiev [97] carried out theoretical nanopore-based sensing of the folding state of a protein. They showed that nanopores can be utilized to detect folding-unfolding transitions in real time and analyzed the structure of folding intermediates through MD simulations. We should emphasize that nearly all the current studies infer the folding process indirectly by depending on the assistance of denaturants, which may differ from the actual folding process of the proteins. Probing dynamic folding processes using nanopores directly instead of inferring the folding process with the assistance of denaturants remains challenging.

3.3. Detection of modifications in single proteins

Posttranslational modifications (PTMs) of proteins, such as phosphorylation and ubiquitination, play crucial roles in a myriad of biological processes. Because a series of PTMs are often required to convert proteins into functional, mature molecules, there is no doubt that nanopore-based studies on protein modifications will accelerate our understanding of these fundamental biological mechanisms.

Detection of protein phosphorylation is one of the focuses of Bayley's team. In 2014, they demonstrated site-specific detection of protein phosphorylation with α-HL at the single-molecule level. In their experiments, thioredoxin was phosphorylated at two adjacent sites. Through analyzing the distinctions in the electrical

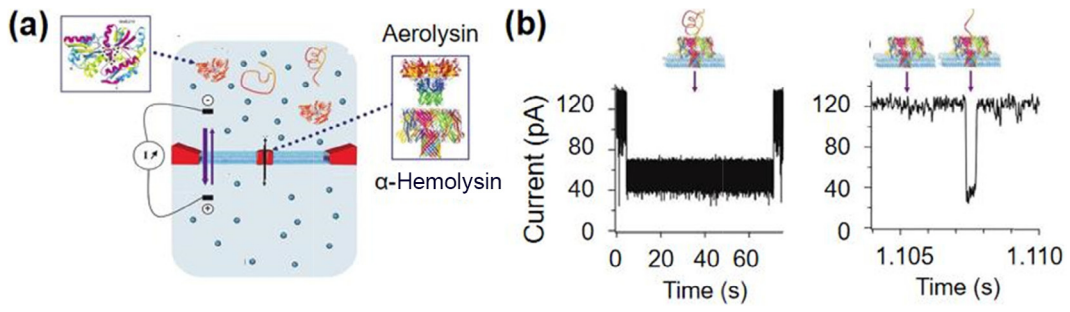

Fig. 3. The unfolding processes of a single protein with the aid of Gdm-HCl. (a) Principle of protein conformation detection using biological nanopores. (b) Ionic current signal of partially folded protein and completely unfolded protein. Reproduced with permission from Ref. [89]. Copyright 2012, American Chemical Society.

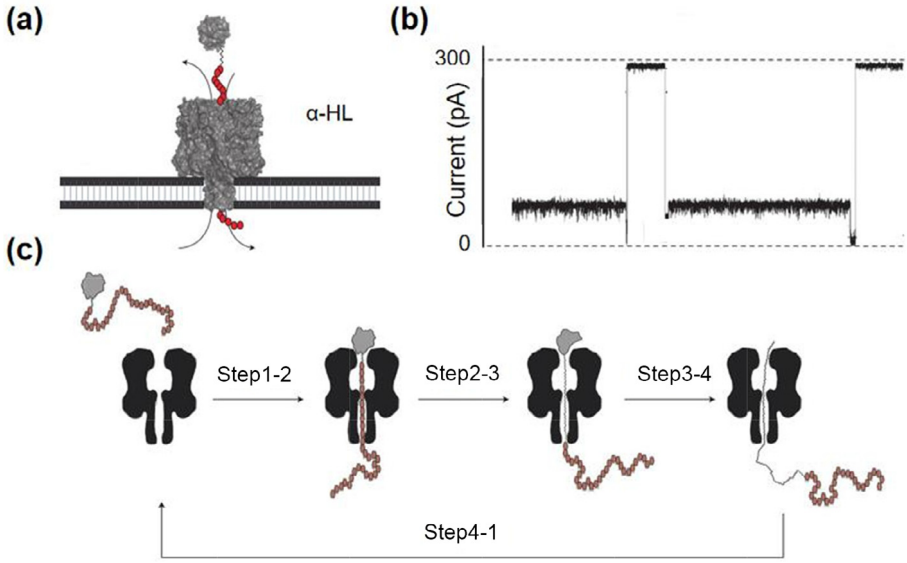

Fig. 4. The unfolding processes of a single protein. (a) Schematic diagram of protein unfolding detection using biological nanopores. (b) A current signature example for an unfolding protein. (c) Schematic diagram of the four-step unfolding process. Reproduced with permission from Ref. [92]. Copyright 2013, Nature Publishing Group.

translocation signals of thioredoxin, they successfully recognized unphosphorylated, monophosphorylated and diphosphorylated variants [98]. Furthermore, in 2018, the group examined reversible protein phosphorylation caused by protein kinases and phosphatases. Phosphorylation and dephosphorylation of a single protein was observed using biological nanopores. They also investigated the effect of divalent cations (Mg^{2+} and Mn^{2+}) on binding between a substrate and product [99]. In 2017, Wloka et al. [100] employed an engineered Cytolysin A (ClyA) nanopore to detect the ubiquitination of proteins. Relying on statistical data, monoubiquitinated and polyubiquitinated forms of proteins could be discriminated through ionic current signals.

4. Protein-protein interactions

Protein-protein interactions (PPIs) are essential for many cellular processes. In nanopore-based studies, PPIs such as antigen–antibody and ligand-receptor interactions have been investigated. In 2013, Freedman et al. [101] detected the interactions between gp120 and its antibody, differentiating monovalent and multivalent binding of gp120 and anti-gp120 antibody based on electrical measurements. In addition, Kim's group [102,103] carried out several studies on the interactions between p53 transactivation domain (p53TAD) and mouse double minute 2 (MDM2). Via separate detection of the translocation signals of p53TAD and MDM2, it was found that the two proteins possess opposite electrical charges in neutral buffer, but the MDM2-p53TAD complex was negatively charged (Fig. 5a). Additionally, it was shown that the double-peak signal characteristic of the MDM2-p53TAD complex disappeared after addition of nutlin-3 to separate MDM2 and p53TAD (Fig. 5b, c). In 2018, Thakur and Movileanu [104] observed ionic current transitions upon binding of the protein ligand ferric hydroxamate uptake component A to its receptor the mutated RNase barnase. The reversible capture and release process could be monitored in real time from the current traces.

Studies related to the interactions between proteins and biological nanopores have also been conducted. For instance, Fahie et al. [105] showed in 2015 that two anti-biotin antibodies could be individually or simultaneously discriminated by biological nanopores. The conductance of nanopores was affected by the position of the binding site through interactions between antibodies and biological nanopores. Thus, dynamic PPIs can be probed in real time with the assistance of powerful nanopore methods.

5. Protein-DNA interactions

In vivo, a DNA molecule almost always exists and functions in complex with proteins. In this section, studies using nanopores to decipher the mechanisms of nonspecific protein-DNA binding and specific protein-DNA binding are discussed.

5.1. Nonspecific binding between proteins and DNA

Nanobeads and optical tweezers are widely used to assist measurements of nonspecific protein-DNA binding in nanopores. In this context, double-stranded DNA (dsDNA) can be covalently attached to nanobeads, whose position can be controlled by optical tweezers. For example, binding of RecA with dsDNA significantly impacts both the force spectra and the ionic current fluctuation, which can be utilized to analyze interactions between RecA and dsDNA (Fig. 6) [106,107]. On the basis of this system, Venkatesan et al. [108] in 2012 employed a graphene-Al_2O_3 nanopore to distinguish single dsDNA molecules and RecA-coated DNA based on their resistive pulses. In 2015, Marshall et al. [109] used a solid-state nanopore to study the binding of *E. coli* single-stranded binding protein (SSB) to single-stranded DNA. They found that the SSB/ssDNA complex could be distinguished from bare ssDNA and SSB according to the current signatures (Fig. 7).

5.2. Specific binding between proteins and DNA

Specific binding between proteins and DNA usually depends on recognition of specific DNA sequences (aptamers, for example) or various tag modifications on DNA (such as biotin and streptavidin). Below, some of the studies that employ nanopores to study this important interaction are described.

5.2.1. Specific interactions between proteins and ssDNA/dsDNA

The earliest studies on specific interactions between DNA and proteins using nanopores primarily focused on the binding force between DNA and endonuclease/exonuclease. In 2007, Zhao et al. [110] analyzed the interactions between a restriction enzyme

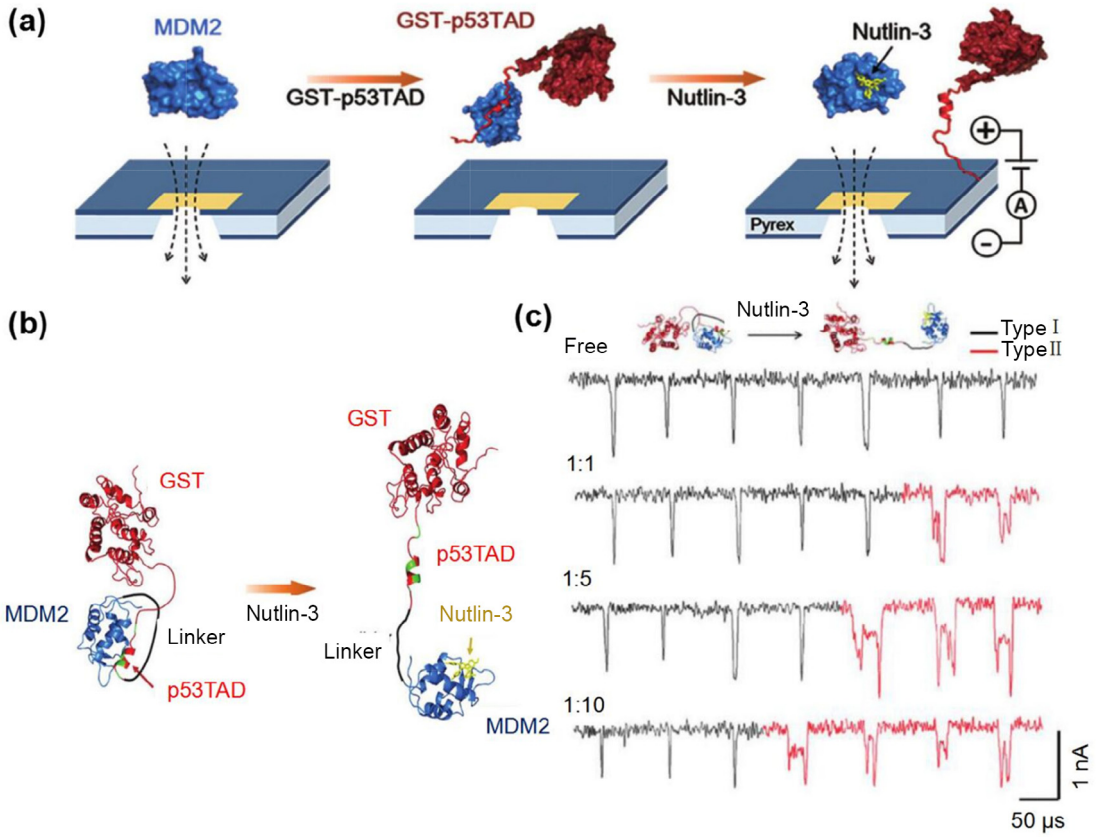

Fig. 5. The interactions between proteins. (a) Diagram of MDM2/GST-p53TAD translocation before and after the addition of nutlin-3. Reproduced with permission from Ref. [102]. Copyright 2016, John/Wiley & Sons, Inc. (b) Two types of current signatures caused by two states of the MDM2/GST-p53TAD complex. (c) Examples of two types of current signals produced as the concentration of nutlin-3 was increased. Reproduced with permission from Ref. [103]. Copyright 2018, RSC Pub.

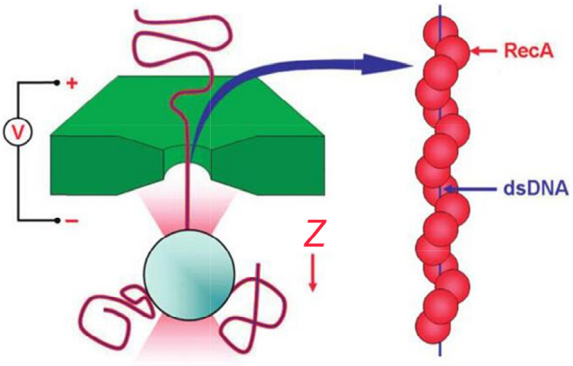

Fig. 6. Schematic diagram of nanobeads controlled by optical tweezers and RecA-coated dsDNA. Reproduced with permission from Ref. [106]. Copyright 2009, American Chemical Society.

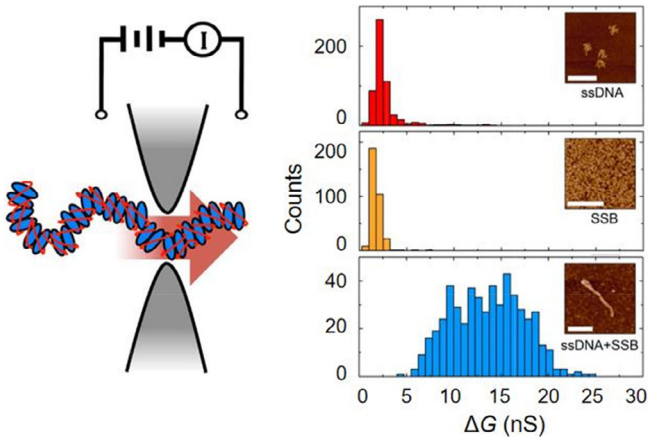

Fig. 7. Histograms for ssDNA, SSB and ssDNA + SSB. Reproduced with permission from Ref. [109]. Copyright 2015, American Chemical Society.

and DNA, discovering that sufficiently high voltage will rupture the DNA-protein complex. By measuring the threshold voltage with a nanopore, a single mutation in the binding sequence of the restriction enzyme could be detected. Based on this work, Dorvel et al. [111] qualitatively compared the stability of bonds in the restriction enzyme-DNA complex. Similar to Zhao's work, Hornblower et al. [112] in 2007 measured interactions between ssDNA and exonuclease I using a voltage-driven method. Despite these advances, we should mention that challenges arising from the stochastic processes can cause variations and nonspecific interactions between DNA and nanopores and that finding a reasonable interpretation of nanopore force spectroscopy measurements remains difficult [113].

Proteins can also bind nucleic acids possessing unique structures (i.e., aptamers). Accordingly, proteins and their putative binding aptamers have been employed in nanopore-based detection. In 2009, Ding et al. [114,115] decorated the sensor of a solid-state nanopore with aptamers. By monitoring the stepwise blockades of the ionic current trace caused by binding of the aptamers and target molecules, they could distinguish single immunoglobulin E and the bioterror agent ricin. In 2014,

Mahmood et al. [116] explored the interactions between thrombin-specific G-quartet aptamers and thrombin based on MD simulations. They linked the aptamers to the inner wall of a nanopore to enable the overall potential energy spectrum to change as target proteins (thrombin) interacted with the aptamers. In the same year, Meervelt et al. [117] carried out a similar experimental study by using ClyA nanopores to study the interaction between the G-quadruplex fold of the thrombin binding aptamer (TBA) and human thrombin (HT). In 2017, Sze et al. [118] demonstrated simultaneous nanopore-based detection of multiple protein targets each bound to a specific aptamer hybridizing to a specific segment of a dsDNA (Fig. 8). The proteins bound to the aptamers produced unique local details in ionic current signals, enabling recognition of different numbers of proteins (1, 2 or 3) and of different protein types (thrombin and acetylcholinesterase). Furthermore, this method enabled specific, single-molecule screening in human serum at ultralow protein concentrations. Clearly, implementing aptamers is beneficial to protein detection via nanopores.

5.2.2. Specific interactions between proteins and self-assembled DNA nanostructures

The ability to manipulate and control the behavior of a molecule to be analyzed is crucial for obtaining accurate measurements in nanopore-based assays. One promising approach to accomplish this is DNA self-assembly, which utilizes specific molecular recognition between DNA strands to guide the spontaneous synthesis of stable structures via hydrogen bonding, hydrophobic interactions and van der Waals interactions [119].

To date, several studies have demonstrated the ability of assembled DNA nanostructures to improve nanopore sensors, with the majority of the research progress contributed by Keyser and coworkers. For instance, the group synthesized dsDNA carriers by hybridizing hundreds of DNA oligonucleotides with m13mp18 (an M13 lac phage vector). They demonstrated that the carriers enable covalent binding of specific proteins at a designated position (Fig. 9a, b) [120] and could be used to differentiate protein quantity and species. In 2016, the group further demonstrated the ability of the carriers to realize quantitative protein detection in the nanomolar range [121]. In this work, the fraction of the

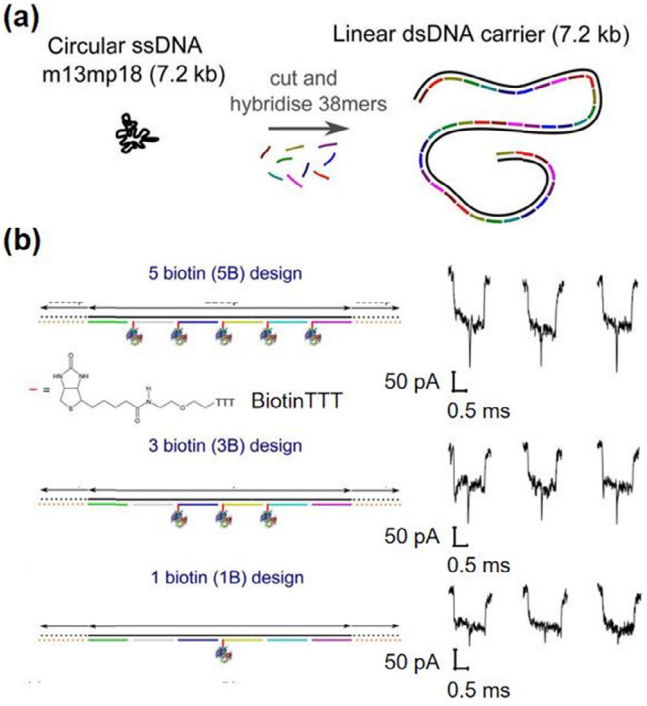

Fig. 9. The utilization of self-assembled DNA nanostructures in characterizing proteins. (a) Schematic of a 7.2 kb DNA carrier. (b) Translocation events of the DNA carrier with 5 bound streptavidin molecules, 3 bound streptavidin molecules and 1 bound streptavidin molecule. Reproduced with permission from Ref. [120]. Copyright 2015, American Chemical Society.

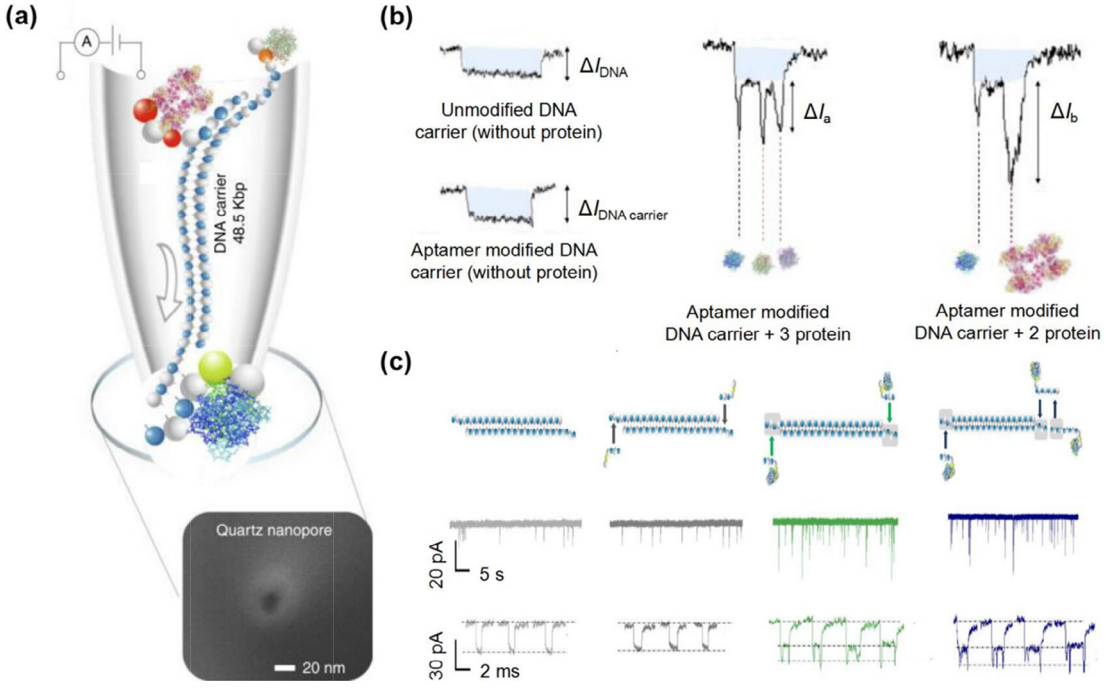

Fig. 8. The screening of proteins using specific aptamers and nanopores. (a) Sketched map of aptamer-assisted protein detection using nanopores. (b) Current signatures for different proteins. (c) Current traces and scatter plots for DNA carriers with different numbers of proteins. Reproduced with permission from Ref. [118]. Copyright 2017, Nature Publishing Group.

secondary resistive pulse of the overall DNA translocation signal was calculated to quantify the concentration of a corresponding protein mixed with proteins of known concentrations. Furthermore, the group designed and prepared digitally encoded DNA carriers to enable simultaneous detection of different proteins [122]. Specifically, each DNA carrier was chemically modified with a unique antigen (e.g., biotin) and hybridized to DNA hairpins at one or more locations that define the barcode of the carrier. By allowing the hairpins to hybridize at up to three locations on the carrier, eight different unique signatures could be obtained, enabling the detection of four different antibodies against their corresponding controls at the same time (Fig. 10). Last but not least, the same group also developed a solid-state nanopore-based method to recognize specific DNA motifs [123]. Using DNA methyltransferase, they covalently attached biotin labels at 5′-TCGA-3′ sequence motifs, enabling the position and number of the motifs to be estimated from the secondary ionic current drop caused by bound streptavidin. In addition, a variety of DNA carriers were characterized by determining the position and number of bound streptavidin proteins.

Physiological properties and biological functions of proteins have also been characterized by combining proteins and assembled DNA structures. In 2018, Ketterer et al. [124] attached a nuclear pore complex (NPC) to a DNA origami ring to study the resulting collective behavior using silicon nitride nanopores (Fig. 11). They were able to distinguish different numbers and types of NPCs from the fluctuation and conductance reflected by their ionic current when the origami ring docked onto the nanopore. At approximately the same time, Fisher et al. [125] published a similar work on NPCs.

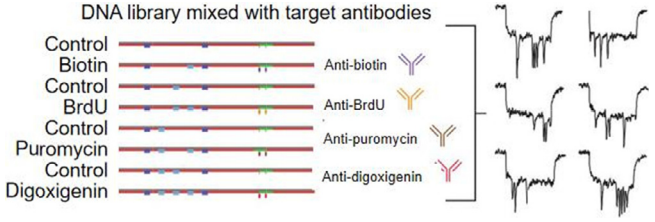

Fig. 10. Schematic of a DNA library mixed with four types of antibodies. The detailed current signatures for the different carriers are also shown. Reproduced with permission from Ref. [122]. Copyright 2016, Nature Publishing Group.

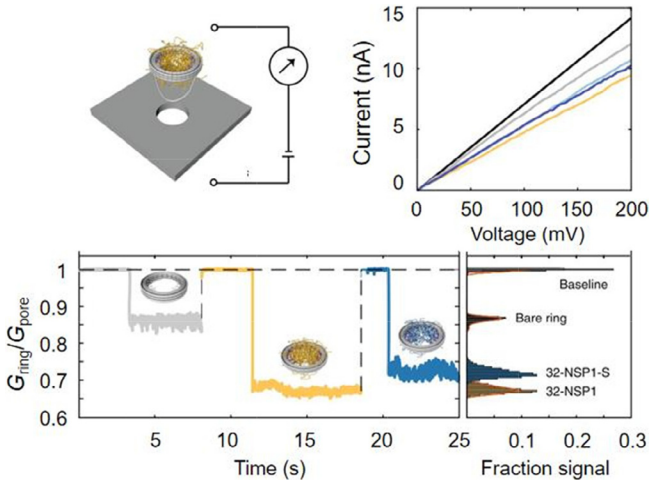

Fig. 11. Study of the nuclear pore complex (NPC) employing a DNA origami structures and nanopore. The differences in the *I-V* curves and current traces are due to different numbers and types of NPCs. Reproduced with permission from Ref. [124]. Copyright 2018, Nature Publishing Group.

In summary, interactions between proteins and ssDNA/dsDNA enable researchers to gain better control over protein translocation through nanopores. Additionally, various protein-DNA assembly complexes have been developed to enable single proteins to be characterized.

6. Conclusion

Proteins are indispensable components of all biological processes. Therefore, the ability to fully characterize protein functions and activities should offer tremendous opportunities for biological and disease studies. Toward this goal, nanopore-based methods for protein analysis have been developed, enabling label-free, single-molecule detection of a specific protein not attainable by commonly employed techniques. Despite great promise, many challenges still need to be overcome in order to provide true and in-depth comprehension of protein properties and activities using nanopore methods. For example, the resolution and controllability of nanopore-based methods need further improvement when applied to the detection of small molecules. Additionally, obtaining a full dynamic view of the conformational changes of a single protein under physiological conditions remains challenging. While 2D or 3D self-assembled DNA structures have shown great promise in significantly improving the performance of nanopore-based methods, more work is needed to enable better interfacing between the DNA-assembled nanostructures and nanopores. With further refinement of nanopore design and development of more sophisticated measurement and fabrication techniques, we envision that nanopore-based approaches could promote new discoveries regarding protein functions and activities.

Conflict of interest

The authors declare that they have no conflict of interest.

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Author contributions

Taoli Ding, Antony K. Chen and Zuhong Lu wrote the manuscript. All authors read and approved the manuscript.

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